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The goal of this research project is to determine how CD44, the cell surface receptor for hyaluronan, stimulates tumor progression. Based upon a variety of evidence, we postulate that CD44 allows tumor cells to degrade hyaluronan which, in turn, increases their vascular supply. During this past year, we have prepared a series of human breast cancer cell lines that have been transfected with a CD44 expression vector. In the immediate future, we will inject these CD44 expressing cells into nude mice and determine if the resulting tumors demonstrate an increased number of endothelial cells as compared to tumors from control cells that do not express CD44. In another set of experiments, we have examined a series of human breast cancer cell lines that differ in their invasive properties (MCF10A before and after transfection with the oncogenes c-HA-ras and/or c-erbB-2). We found that the expression of the low molecular weight isoform of CD44 (85 kDa), but not the high molecular weight isoforms (105-150 kDa), was correlated with the invasive properties of the cells and their ability to bind isotopically labeled hyaluronan. These results suggest that the expression of the 85 kDa isoform of CD44 may be the best marker of hyaluronan binding and degradation, as well as the metastatic behavior of tumor cells.

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Charla Lindenhill 9/23/95

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INTRODUCTION:

Nature of the Problem:

The present research project is concerned with the interaction between tumor cells and hyaluronan, one of the major components of the extracellular matrix. Hyaluronan is a very large, negatively-charged carbohydrate that functions to maintain the extracellular space. In previous studies, we have shown that the degradation of hyaluronan (HA) is mediated by a cell surface glycoprotein termed CD44 (also known as the hyaluronan receptor). This protein functions to bind hyaluronan to the cell surface so that it can be internalized and then degraded by lysosomal enzymes. We have found that this degradatory process can be prevented by antibodies which block the interaction between CD44 and hyaluronan.

The working hypothesis of the present application is that this CD44-mediated degradation of hyaluronan enhances tumor progression by increasing their vascular supply. This hypothesis is supported by the following lines of evidence. First, a number of studies have shown that the expression of CD44 is causally associated with the metastatic process. For example, transfection of cells with CD44 expression vectors stimulates their metastatic properties. Secondly, human breast cancer cell lines that express CD44 can degrade hyaluronan. Thirdly, the fragments of hyaluronan produced in the process of degradation have angiogenic properties leading to increased vascularization. And fourthly, large amounts of hyaluronan surround many types of blood vessels, and the degradation of this hyaluronan by tumor cells would increase their vascular supply.

Background of Previous Work:

General Characteristics of CD44: CD44 defines a family of cell surface glycoproteins which has been implicated in cellular processes such as adhesion, migration, lymphocyte homing and tumor metastasis (1, 2). These proteins are found on a variety of cell types including epithelia, leukocytes, and tumor cells. As a result of alternative splicing and variations in the degree of glycosylation, members of the CD44 family come in several different molecular weight forms, ranging from 80 to well over 200 kDa (2).

As illustrated in *Fig. 1,* CD44 may be divided into three domains, base upon both structural and functional considerations. First, the C-terminal domain of the molecule consists of the transmembrane and cytoplasmic region of the molecule. This region of the molecule can be associated with actin filaments, possibly through an ankyrin-like molecule, and this interaction may be modified by either phosphorylation or acrylation (6-8). The association with the cytoskeleton may be an important factor in determining the distribution of CD44 on the cell surface which, in turn, may influence it ability to interact with HA. Secondly, the middle domain of the molecule is highly glycosylated and in some cases may serve as an attachment site for either

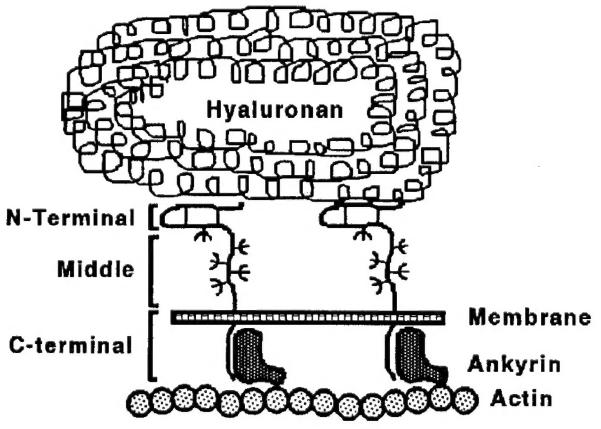

Fig. 1. Model of CD44 and its interactions with the cytoskeleton and HA.

chondroitin or heparan sulfate side chains, which are responsible for the interactions with collagen and fibronectin (9-11). This region of the molecule shows considerable variation in sequence due to alternative splicing of the mRNA. Already, at least 15 isoforms of CD44 have been identified, and most of the different inserts occur in this middle domain (12). And thirdly, the N-terminal domain shares sequence homology with link protein of cartilage and is responsible for the binding of HA. This region recognizes a six sugar sequence of HA, but will also bind chondroitin sulfate with a lower affinity (1, 2).

Involvement of CD44 in Tumor Progression: Recently, several lines of evidence have suggested that CD44 is involved in tumor metastasis. For example, a number of studies have found that high levels of CD44 are associated with certain types of carcinomas, high grade gliomas and many non-Hodgkin's lymphomas (17-19). In the case of lymphomas and other tumors, large amounts of this protein are correlated with the rapid dissemination and negative prognosis of these tumors (18, 19). In preliminary studies, we have also found that the expression of CD44 by a panel of human breast cancer cell lines is correlated with their metastatic behavior as measured by a variety of *in vitro* assays.

More direct evidence that the expression of CD44 is related to the metastatic behavior of tumor cells comes from the work of Gunthert and his associates (20). They found that highly metastatic rat pancreas cell lines express a particular isoform of CD44 (termed CD44v), which was absent from their non-turmorigenic counterparts. More importantly, when non-metastatic cells were transfected with cDNA for this CD44 isoform, they were converted into a more metastatic phenotype (20). In addition, antibodies directed against this particular isoform of CD44 blocked tumor metastasis in experimental models (20). These observations suggest that CD44v is responsible for the metastatic behavior of these cells.

Other isoforms of CD44 also appear to influence the metastatic behavior of cells. Sy et al. (21) have shown that when human lymphoma cells were transfected with the cDNA for a 85 kDa isoform of CD44 which binds HA, there was a marked increase in tumor formation and metastatic behavior, while transfection with an isoform that cannot bind HA had no such effect. In addition, the growth of these tumors *in vivo* could be blocked by co-injection of a soluble form of CD44, which presumably acted by competitively inhibiting the interactions of CD44 with its ligand, HA (22). These researchers also noted that lymphoma cells lacking CD44 also formed both primary and metastatic tumors, albeit at a lower rate. Based on these results, these researchers concluded that expression of the 85 kDa form of CD44 promotes, but is not required for, tumor growth and metastasis (21).

However, none of the studies described above address the mechanism by which CD44 promotes tumor progression. This question is the major goal of the present research project.

Role of CD44 in Degradation of HA: One possible mechanism by which CD44 could influence the behavior of tumor cells is by mediating the degradation of HA. Indeed, in earlier studies, we have shown that CD44 is critically involved in the uptake and degradation of HA by both transformed fibroblasts (SV-3T3 cells) and alveolar macrophages (23). To demonstrate this phenomenon, we cultured these cells in the presence of [^{3}H] HA. After various lengths of time, the cultures were digested with pronase to release the HA, and the fragments of [^{3}H] HA were separated from the macromolecular HA by centrifugation through size specific membranes (Cetricon 30 Micro concentrators). Both the SV-3T3 cells and the macrophages degraded significant amounts of the HA.

Examination of the digests by molecular-sieve chromatography revealed that the resulting fragments ranged in size from monosaccharides to higher oligosaccharides; smaller fragments were not detected.

CD44 was clearly involved in the degradation of HA, since this process was almost completely blocked by the K-3 mAb against CD44. Furthermore, the degradation was also blocked by the addition of an excess of non-labeled HA, while the addition of other glycosaminoglycans such as dermatan sulfate, chondroitin-4-sulfate and heparin had only a small inhibitory effect (23). This was in keeping with previous studies indicating that CD44 binds with relative specificity to HA as compared to other glycosaminoglycans (1). Similarly, oligosaccharide fragments of HA smaller than a hexasaccharide had only a modest inhibitory effect on the degradation, which is consistent with the size specificity for recognition by CD44 (1).

Collectively, the above results indicated that CD44 plays a key role in the degradation of HA. More specifically, CD44 is responsible for the initial binding of HA to the cell surface so that it can be internalized and degraded by acid hydrolases (see model in *Fig. 2*). This CD44-mediated uptake is consistent with previous studies suggesting that CD44 is associated with the cytoskeleton (6). Thus, the degradation of HA takes place in a fashion similar to that of other receptor-mediated degradatory processes such as LDL and transferrin.

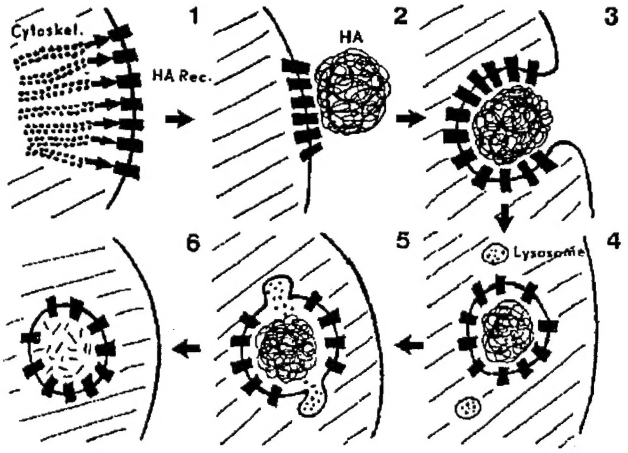

Fig. 2. Model of HA degradation. (1) Initially molecules of CD44 are clustered on the cell surface through their interaction with the cytoskeleton. (2) A number of molecules of CD44 bind simultaneously to a molecule of HA. (3 and 4) The HA is endocytosed into a vesicle. (5) Lysosomes fuse with the vesicle. (6) The HA is degraded by the action of acid hyrolases.

The ability of cells expressing CD44 to degrade HA may be important during normal processes of tissue morphogenesis and cell migration. For example, during the development of the lungs, there is a progressive decrease in the amount of HA in relation to protein content (24). The decrease reflects the loss of interstitial tissue so that gas exchange can take place at the time of birth. We found that this loss of HA was inversely correlated with the number of macrophages expressing CD44, which increased in number during embryonic development. In addition, histochemical staining revealed that some of these macrophages contained HA in their cytoplasm, suggesting that macrophages had internalized HA from the extracellular matrix. This possibility was further supported by the fact that when new-born mice were injected with the KM-201 monoclonal antibody, which blocks the interaction between HA and mouse CD44, the number of HA-containing macrophages in the lungs decreased while the concentration of HA increased. Taken together, these results suggest that macrophages can internalize HA during lung development and could possibly play a significant role in its removal (24).

CD44 and HA of Human Breast Cancer Cell Lines: In preliminary studies, we have examined the relationship between CD44 expression and the binding and degradation of HA in a panel of human breast cancer cell lines (26). These cell lines have been previously characterized for various markers of

invasive potential and represent a convenient *in vitro* model system for studies of breast cancer progression (27).

In general, the cell lines that expressed the most CD44 were also the most invasive, as judged by *in vitro* assays. For example, the Hs578T cell line that expressed the greatest amount of CD44 was invasive, as judged by migration and chemotaxis in Boyden chamber assays, while the ZR-75-1 cell line, that did not express detectable levels of CD44, was judged to be non-invasive in both of these assays. Similarly, the expression of high amounts of CD44 was generally associated with the lack of estrogen receptors and the presence of the intermediate filament protein vimentin, both of which have been shown to indicate a poor prognosis in human breast cancer (27). This trend is consistent with other studies indicating that the expression of CD44 is correlated with metastatic behavior of tumor cells (17-19)

We then examined the ability of these cells to degrade HA. For this, the cells were cultured in the presence of [^{3}H]HA, and after 40 hours, the resulting fragments were detected using Centricon 30 micro concentrators. The degradation of HA was closely correlated with the amount of CD44 (correlation coefficient, $r = 0.951$). In general, the cell lines that expressed the most CD44 also could degrade the most HA. This correlation was remarkably good, considering the fact that other factors are clearly involved in the degradation process, such as the rate of endocytosis and the amount of lysosomal HAase.

The involvement of CD44 in HA degradation was further supported by the observation that Hermes-1 mAb, which is directed against an epitope close to the HA binding domain of CD44 (3), blocked the degradation of HA.

We then examined the distribution of HA in xenografts formed by these cell lines in nude mice. For this, the cell lines were injected into the fat pads of nude mice, and the resulting xenografts were histochemically stained for HA, using a specific probe derived from cartilage. One feature common to all of the grafts was that HA was a prominent component of the matrix at the junction between the graft and the surrounding normal tissue. In some cases, the demarcation boundary between graft and the surrounding tissue was diffuse, while in others it was relatively sharp. The type of boundary differed both from tumor to tumor and within a single tumor.

Significant differences were observed in the distribution of HA within the body of the tumor xenografts. In the grafts of cells that expressed low levels of CD44, HA was generally a major component of the interstitial matrix. In contrast, in the body of grafts formed by cells that expressed high levels of CD44, HA was greatly reduced or absent. These grafts were relatively deficient in interstitial connective tissue and had a more homogenous appearance. The one exception to this correlation was the MDA-468 cell line, in which the amount of HA varied significantly from region to region. However, in general, the expression of CD44 was inversely correlated with presence of HA within the body of the tumor cell xenografts. We speculate that this difference is due to the ability of CD44 expressing tumors to degrade the HA.

Effect of HA Degradation on Vascularization: The central question being addressed in this research project is how does CD44 enhance the metastatic activity of tumor cells. Based upon a variety of evidence, we speculate that the CD44-mediated degradation of HA lead to an increase in the blood supply to the tumor cells which enhances their growth rate as well as their ability to survive and form

metastases. This postulated increase in blood supply may occur through two different mechanisms, which may occur simultaneously.

First, the oligosaccharide fragments of HA produced as a by-product of HA degradation may stimulate the formation of new blood vessels. Indeed, studies have shown that oligosaccharide fragments of HA have angiogenic properties. For example, West and coworkers found that fragments of HA 3 to 16 disaccharides in length stimulate the formation of blood vessels when applied to the chick chorioallantoic membrane (28). In contrast, macromolecular HA and fragments of other glycosaminoglycans (chondroitin-4 and 6-sulfate) were ineffective, suggesting that the effect is specific for HA. These workers went on to show that these oligosaccharide fragments of HA also stimulated the proliferation of endothelial cells in tissue culture (29). This effect appeared to be restricted to endothelial cells since fibroblasts and smooth muscle cells were not effected by these fragments. Presumably, the endothelial cells contain a receptor that can detect fragments of HA. This receptor is probably distinct from CD44 which in most cases is not present on endothelial cells. Along these lines, Banerjee and Toole (30) have shown that antibodies against an HA binding protein on the surface of endothelial cells blocks the migration of these cells. Thus, it is possible that tumor cells expressing CD44 could release fragments of HA which interacts with receptors on the surfaces of endothelial cells and stimulate the formation of new blood vessels.

A second possible mechanism is that tumor cells expressing CD44 can degrade the HA surrounding blood vessels. In histochemical studies, we have examined the distribution of HA surrounding blood vessels in different tissues. In some tissues, such as the liver and spleen, only small amounts of HA are associated with the blood vessels. In contrast, in other tissues such as the dermis, the lamina propria of the intestinal track, the stroma of the lungs and the pericardium of the heart, large amounts of HA are associated with the blood vessels. In these tissues, HA was generally associated with the intima of veins and venules, immediately beneath the endothelial cell lining. In contrast, in arteries, it was generally reduced or absent from the intima, but was present in the adventitia. Thus, the ability of tumor cells to degrade this HA could allow them to get in closer proximity to the blood supply and consequently receive more nutrients. Along these lines, it is also possible that these tumor cells could more easily penetrate the blood vessels, enter the circulation and metastasize to different locations.

We further hypothesize that regardless of the mechanism, the increase in the blood supply results in a selective advantage for those cells that express CD44. When we stain normal mouse mammary tissue for CD44, we find that only small amounts of it are expressed on the ductal cells. However, in primary tumors of transgenic mice, we find that the expression of CD44 is variable. It is present in some regions but absent from others. We speculate that the CD44 expressing cells of the primary tumor are at a selective advantage for giving rise to metastases. One of the specific aims of this research project is to determine if the metastases that arise from these mixed primary tumors have a high probability of expressing CD44.

Purpose of the Present Work:

The working hypothesis of this proposal is that the expression of CD44 allows tumor cells to degrade HA, which, in turn, results in an increase in the blood supply. This increase may occur by formation of new blood vessels induced by fragments of HA, and/or by the degradation of HA present around preexisting blood vessels, which improves the access of the tumor cells to the blood supply. In either case, the

increase in the blood supply imparts a selective advantage to the tumor cells that express CD44. As a result, while primary tumors may be heterogeneous with respect to the expression of CD44, secondary tumors will have much higher probability of expressing this molecule.

Methods of Approach:

1. Examine the effect of CD44 expression of the vascularization of tumors: To determine if the expression of CD44 leads to an increase in the vascular supply, we will transfect a human breast cancer cell line (ZR-751) with a CD44 expression vector and select cells that stably express this protein. Both CD44 positive and negative cells will be injected into nude mice and allowed to grow. The resulting xenografts will be surgically removed and examined histologically for the presence of blood vessels. If our hypothesis is correct, then xenografts derived from CD44 positive cells should be associated with a greater number of blood vessels than the CD44 negative cells.

2. Determine the effects of various agents on the vascularization of tumors expressing CD44: Osmotic pumps that release either control or blocking antibodies to CD44 will be implanted subcutaneously in nude mice along with tumor cell lines that express CD44. After a period of growth, the xenografts will be removed and examined histologically for blood vessels. If our working hypothesis is correct, then the blocking antibodies should inhibit the vascularization of the tumor cells. In a similar experiment, the osmotic pump will be filled with oligosaccharide fragments of HA or HAase, which should enhance vascularization of tumor cells that do not express CD44 if the working hypothesis is correct.

3. Examine the expression of CD44 in primary and secondary tumors of transgenic mice: According to our working hypothesis, the expression of CD44 imparts a selective advantage to cells with regard to tumor progression. This suggests that while primary tumors may be mixed with respect to the expression of CD44, the secondary tumors (i.e. metastases) will be selected from the CD44 positive sub population. To examine this possibility, we will examine both primary and secondary tumors formed by transgenic strains of mice that spontaneously develop breast tumors. The xenografts will be analyzed histochemically for endothelial cells.

4. Survey specimens of human breast tumor for the presence of CD44, HA and vascular endothelial cells: To determine the significance of CD44 and HA in evaluating its metastatic potential, we will examine specimens of human breast cancer, which are available from the tumor bank of the Lombardi Cancer Center. The specimens in this collection will have been classified by various criteria including survival of the donor. In this pilot study, we will analyze the presence of CD44 and HA in subsets of these tumor specimens with respect to metastatic versus non metastatic (node positive vs. node negative) and short versus long survival term survival of the donor. If this pilot experiment shows a good correlation between these parameters then we will expand this study to include a greater number of samples.

BODY

During the first year of this project, we have devoted most of our time to the preparation of reagents to carry out the proposed studies. The progress that has been make on each of the tasks will be described in the following sections.

Task 1: Examine the effect of CD44 expression of the vascularization of tumors: The purpose of this study is to test our working hypothesis that CD44 enhances the vascularization of tumors. To accomplish this, we have transfected a human breast cancer cell line with a CD44 expression vector. These cells will then be grown in nude mice, and the resulting xenografts will be analyzed histochemically for endothelial cells.

Preparation of Plasmid Vector: A cDNA clone of the full length human CD44 (pBL32) was obtained from Dr. Butcher (Stanford, CA) and consists of a 2.2 Kb insert in pUC118 (31). The plasmid was digested with *XbaI* and *Hind III*, and the insert was then ligated into the polylinker region of the expression vector pRc/CMV (InVitrogen, San Deigo, CA). This vector was chosen over the pCMV5 which was proposed in the initial application because it also contains a neomycin resistance gene which greatly simplifies the selection process. The plasmid (pRc/CMV-CD44) was then transfected into competent DH5α cells for amplification and finally purified by the triton lysis technique. Prior to transfection, the plasmid was linearized by treatment with *Bgl II*.

Transfection of Cultured Cells: The human breast cancer cell line ZR-751 that does not express CD44 was cultured in 35 mm plates in 10% fetal calf serum, 90% DMEM. Immediately prior to transfection, the medium was removed from the cultured cells (50% confluent) and replaced with 1 ml of Opti-MEM medium (GIBCO-BRL). To this medium was added a mixture of 5 μg of the linearized plasmid with 50 μl of lipofectamin (GIBCO-BRL) and the cells were incubated for 24 h at 37°C. The transfection medium was then replaced with 3 ml of 10% fetal calf serum, 90% DMEM and the cells were grown for 48 h. The selection was then carried out using G-418 sulfate (geneticin) at a concentration of 10 μg/ml for the first 2 days, and there after at 1 mg/ml. After 10 to 15 days, the non-transfected cells had died, leaving clones of resistant cells. The resistant clones were further grown in the presence of geneticin until they reached a size large enough to be transferred. Eight positive and two control clones were then isolated using glass rings and trypsin and expanded in culture. These clones were then tested for the expression of CD44 using an antibody against CD44 (b-BU-52). Three of the pRc/CMV-CD44 transfected clones expressed large amounts of CD44 as measured by immunostaining. In contrast the control (parent) cells were negative for CD44.

Assay of HA Binding and Degradation: To make sure that the transfected ZR-751 cells are functionally active, they will be examined for both HA binding and degradation activity (23).

The [^{3}H] HA to be used in these assays was prepared by growing *R*at fibrosarcoma cells in the presence of [^{3}H] acetate in complete medium (10% fetal calf serum, 90% DMEM). The medium was collected, digested with pronase and dialyzed extensively against distilled water. The [^{3}H] HA was purified by precipitation with cetylpridinium chloride and then re dissolved in a saline solution. The amount of HA is determined by an uronic acid assay (32, 33). The preparation used in this study had a specific activity of 7.6×10^5 cpm/μg of Na hyaluronan.

For the binding assay, detergent extracts of the cells will be mixed with the [^{3}H] HA and then an equal volume of saturated $(NH_4)_2SO_4$ will be added. Following centrifugation, the precipitated CD44 and the bound [^{3}H] HA will be analyzed by scintillation counting.

For the degradation assay, the cells will be cultured in a 24 multi well plate to which is added 2 μg/ml of [^{3}H] HA. After 48 hours, the cultures will be digested with pronase E and applied to Centricon 30 Microconcentrators. Following centrifugation, the material passing through the membrane is collected and processed for scintillation counting (23). The background level of degradation (i.e. in the absence of cells) will be subtracted from each value.

Once the appropriate cell lines have been selected they will be injected into the mammary fat pads of nude mice (NCr nu/nu athymic, ovarectomized, Charles River) along with an estrogen implant. After 4 to 8 weeks, the mice will be given an i.p. injection of pentobarbital (Nembutal, 90 mg/kg body), and then sacrificed by exsanguination. The resulting tumor mass will be fixed and processed for histology. The sections will be stained for 1) CD44 using either the Hermes-1 or BU52 monoclonal antibodies; 2) HA using the b-PG binding probe: and 3) endothelial cells, using a monoclonal antibody to mouse PECAM-1 (Pharmingen). This latter agent was found to be more specific for mouse endothelial cells than the lectin *Dolichos Biflorus agglutinin* that was initially proposed (preliminary studies). From the results of this staining, we will quantitate the number of endothelial cells by counting random fields at the periphery of the xenografts. If our working hypothesis is correct, then a greater number of endothelial cells and blood vessels will be associated with the xenografts expressing CD44, than with those that do not. If no such association is observed then we will reassess the initial working hypothesis.

Task 2. Determine the effects of various agents on the vascularization of tumors expressing CD44: The purpose of this set of experiments is to determine if xenograft vascularization can be blocked by antibodies to CD44 or enhanced by fragments of HA or HAase. We have just begun these experiments and have isolated the following reagents which will be tested in the future.

Hermes-1 mAb and Fab fragments: The Hermes-1 is a rat mAb directed against human CD44 which blocks its ability to interact with HA (3). The ascites fluid from the Hermes-1 hybridoma was prepared by a commercial laboratory (Bioproducts for Science, Indianapolis, IN). The Hermes-1 mAb was purified from the ascites fluid using the Econo-Pac serum IgG purification kit from Bio-Rad. IgG was also purified from rat serum to serve as a control. The Fab fragments were prepared by digesting the purified mAb with immobilized papain according to the method provided by the Pierce Chemical Company. The Fab fragments were separated from the Fc fragments by chromatography and found to be pure by SDS-PAGE analysis (40). The control for this agent consists of Fab fragments of IgG from whole mouse serum.

Oligosaccharide Fragments of HA: The oligosaccharides were prepared by a limited digestion of a highly purified preparation of HA (Healon, Pharmacia) with testicular HAase (41). The enzymatic digestion was terminated by placing the sample in a boiling water bath for 10 min. Molecular sieve analysis of the product indicates that it has a molecular weight in the range of 10 to 100 kDa.

HAase: Highly purified preparations of both testicular and *Streptomyces* HAase have been purchased from commercial sources.

The agents described above will be first analyzed for endotoxin and if they are negative, they will be placed into small osmotic pumps. These pumps will then be implanted into Nude mice along with CD44

transfected ZRT-751 cells. After 8 weeks, the mice will be sacrificed the tumors will be examined histologically for HA and endothelial cells as described above. If our working hypothesis is correct then we would anticipate that tumor vascularization will be inhibited by Hermes-1 and stimulated by oligosaccharide fragments of HA and HAase.

Task 3. Examine the expression of CD44 in primary and secondary tumors of transgenic mice: The purpose of this set of experiments is to compare primary versus secondary tumors with respect to the expression of CD44. In preliminary studies, we have found that primary mammary tumors of transgenic mice are heterogeneous with respect to the expression of CD44. In other words, part of the tumor expresses CD44 while other parts do not. Based upon our working hypothesis, we predict that secondary tumors are more likely to be derived from cells in the primary tumor that express CD44 than those that do not. To test this hypothesis, we will examine transgenic mice that spontaneously develop mammary tumors. At this point, we are at the initial stages of this project.

We will use the OncoMouse MM/v-Ha ras mammary carcinoma model. This strain of mice contain the v-Ha-ras oncogene under the control of the mouse mammary tumor virus promoter (43). As a result, female mice develop spontaneous mammary tumor which can metastasize to the liver and/or the lungs (43). Both the primary tumors as well as secondary tumors will be stained for CD44, HA and endothelial cells as described above. If our hypothesis is correct then a large fraction of the secondary tumors will be positive CD44. However, at this stage, we have not been able to locate any secondary tumors, which appear to take some time to develop.

In a related series of experiments, we have examined the expression of CD44 on a series of breast cell lines that vary in their invasive phenotype. These cells were initially derived by Ciadeillo et al (44) from the human breast cell line MCF10A which is considered to be representative of normal cells (i.e. non-tumorigenic). This cell line was transfected by the oncogenes Ha-ras (MCF10A-H), erbB-2 (MCF10A-E) both alone and in combination (MCF10A-HE) (44). The resulting cell lines varied in their metastatic properties as determined by Boyden chamber chemotaxis and chemoinvasion assays, in the following order: MCF10A < MCF10A-H < MCF10A-E < MCF10A-HE (Giunciuglio *et al.* unpublished).

When these cell lines were analyzed for CD44 immunoreactivity by Western blotting, significant differences were observed. As shown in Fig. 3, all of the cell lines expressed both the low (85 kDa) and the high molecular weight (105-150 kDa) isoforms of CD44. However, the proportion of each varied significantly between the different cell types. The parent MCF10A cells expressed predominately the high molecular weight isoforms. In contrast, the metastatic MCF10A-HE cell line expressed mainly the lower molecular weight isoform, and was analogous to that of HCV29T, a highly invasive human bladder carcinoma. The other cell lines (MCF10A-H and MCF10A-E) expressed a pattern of CD44 intermediate between these two extremes. Thus, the expression of the low molecular weight form of CD44 appeared to be directly correlated with the metastatic behavior of the cells.

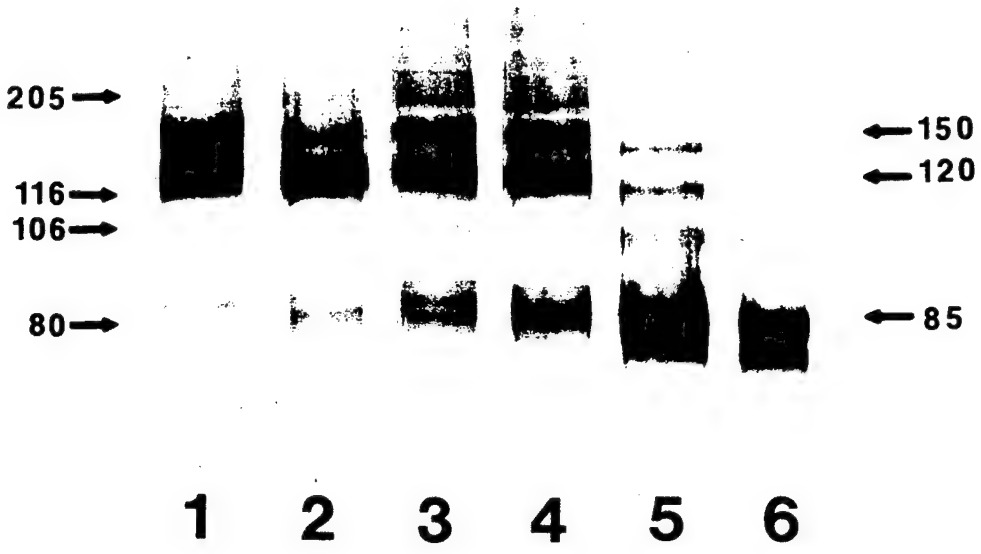

Fig. 3. Western blot analysis of CD44 expression by the parant and transfected cell lines. The cells were extracted in Laemmli sample buffer in the absence of β-ME, run on an 8% polyacrylamide gel and transferred to nitrocellulose. The blot was then stained for CD44 using BU-52 monoclonal antibody. Lane 1, MCF10A (parent); Lane 2, MCF10A transfected with empty vector (null-control); Lane 3, MCF10A-H; Lane 4, MCF10A-E; Lane 5, MCF10A-HE; Lane 6, HCV29T (human bladder carcinoma).

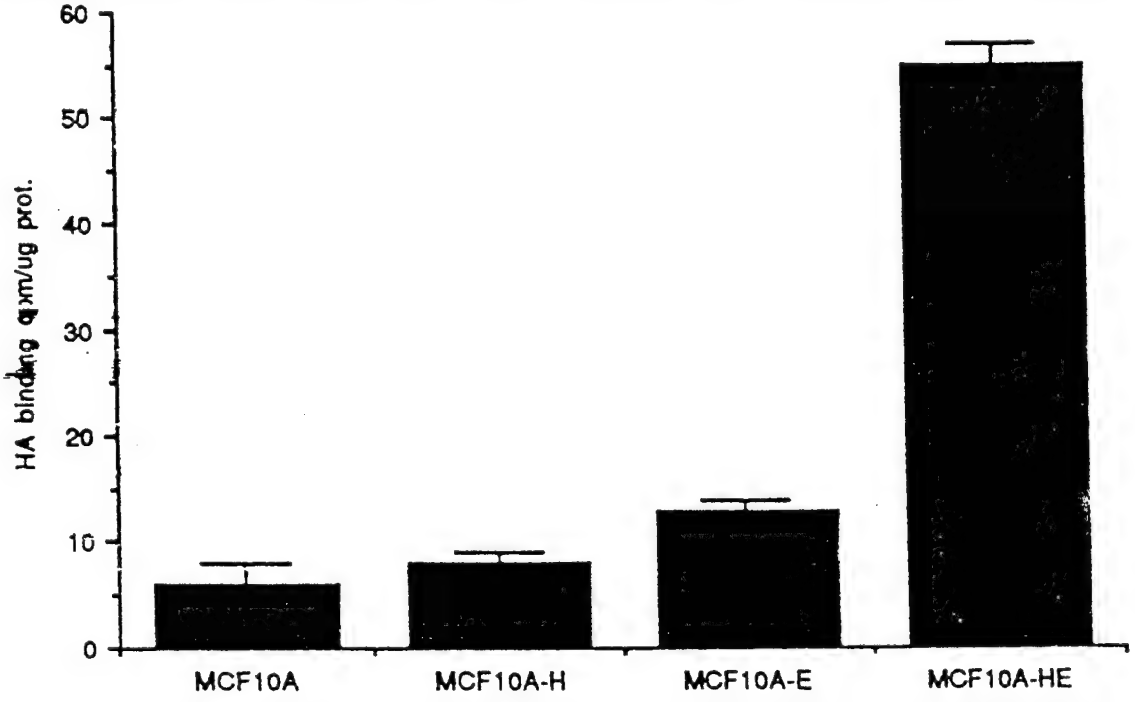

Fig. 4. Binding of [^{3}H]HA by detergent extracts of the cell lines The cells were extracted in a detergent buffer, incubated with [^{3}H]HA and then mixed with $(NH_4)_2SO_4$ to precipitate the HA bound to CD44. The binding is expressed in units of cpm of [^{3}H]HA bound per μg of protein.

We then examined the ability of these cells to bind [^{3}H] HA. As shown in Fig. 4, the parental MCF10A cells as well as the single MCF10A-H and E transfectants bound only modest amouts of HA. In contrast, the doubly transformed MCF10A-HE cells bound 5 to 10 times the amount of HA that was bound by the parent and single transformants. This binding activity is similar to that found in very invasive human breast cancer cell line (26). Thus, the amount of HA binding is correlated with the expression of the 85 kDa form of CD44 as seen in the Western blots.

These results suggest that the expression of the 85 kDa isoform of CD44 confers to the cells the capability to bind HA which, in turn, could allow them to more easily penetrate the blood vessels and metastasize to different locations.

Task 4. Survey specimens of human breast tumor for the presence of CD44, HA and vascular endothelial cells: We propose to carry out a pilot study to determine if the expression of CD44 can be used as a diagnostic indicator of tumor behavior. For this study, we will make use of the Breast Cancer tumor bank which is one of the core facilitates of the Lombardi Cancer Center.

We will begin this particular task in the following year. We are presently testing different antibodies to determine which are the best for detecting human CD44 and endothelial cells in paraffin-embedded tissue sections (note: most of the CD44 antibodies will not react with paraffin sections).

CONCLUSIONS:

Implication of Completed Research:

The major conclusion from the first year of funding is that the 85 kDa isoform of CD44 may be a better marker of a tumor cells' ability to bind HA and to invade through a matrix than the higher molecular weight isoforms. This is an important observation since the antibodies that we use to CD44 recognize all of the different isoforms. Thus, in future studies we may have to distinguish between these different isoforms to get a meaningful correlation.

Recommended Changes:

When examining the cells for CD44 in tasks 3 and 4, we will examine the proportion of both high and low molecular weight isoforms by Western blotting when feasible.

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ACRONYMS AND SYMBOL DEFINITIONS

[^{3}H]HA	Tritium labeled hyaluronan.
b-Hermes-1	Biotinylated form of the hermes-1 monoclonal antibody - used for the localization of human CD44.
b-KM-201	Biotinylated form of the KM-201 mAB
b-PG	Biotinylated proteoglycan - used as specific staining probe for hyaluronan.
CD44	Cluster of determination (differentiation) - same as the hyaluronan receptor or binding site.
CMF-PBS	Calcium and magnesium free phosphate buffered saline.
DMEM	Dulbecco's modifed Eagle's medium
HA	Hyaluronan.
HAase	Hyaluronidase (either testicular or *Streptomyces*)
Hermes-1	Monoclonal antibody against human CD44 - blocks the interaction with hyaluronan.
K-3	Monoclonal antibody against hamster CD44 - blocks the interaction with hyaluronan.
KM-201 mAb	KM-201 monoclonal antibody directed against mouse CD44 - blocks the interaction with hyaluronan.
mAb	Monoclonal antibody.
pCMV5	Expression vector for mammalian cells carrying a cytomegalovirus promoter.
pCMV5-CD44	Expression vector containing sequence for human CD44.
pRc/CMV	Expression Vector containing neomyosin resistance.
pRc/CMV-CD44	Expression Vector containing sequence of CD44.
pSV_2-neo	Plasmid vector which confers neomyosin (G-418 sulfate) resistance - used as a marker for selecting cells that have taken up plasmids (including the PCMV5-CD44)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
SV-3T3	Simian virus 40 transformed mouse 3T3 cells (Swiss mouse).